GENETIC REGULATION OF ALLOLYSIS IN RESPONSE TO ANTIBIOTIC STRESS IN *STREPTOCOCCUS PNEUMONIAE*

THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN

LIFE SCIENCE

By

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411LS2045

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2013

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CERTIFICATE

This is to certify that the project report titled **"Genetic regulation of Allolysis in response to antibiotic stress in *Streptococcus pneumoniae*"** submitted by **Ms. Manisha Dash** to the Department of Life Sciences, National Institute of Technology, Rourkela in partial fulfillment of the requirements for the degree of Masters of Science in LIFE SCIENCE is a bonafide record of work carried out by her under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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ACKNOWLEDGEMENT

I take the privilege to express my utmost gratitude to my guide Dr. Surajit Das, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela for his excellent guidance, care, patience and for providing me with every facility to complete my dissertation. I am also grateful to my faculty members Dr. Bibekanand Mallick, Dr. Sujit Kumar Bhutia, Dr. Bismita Nayak, Dr. Rasu Jayabalan, Dr. Suman Jha for their constant encouragement throughout my dissertation. I am grateful to Dr Samir Kumar Patra, HOD, Department of Life Science, National Institute of Technology, Rourkela for his moral support and valuable advice that kept me motivated throughout my work.

My heartiest thanks to Mr Hirak Ranjan Dash, Phd Scholar, Department of Life Science, National Institute of Technology, Rourkela for his care, co-operation, patience and timely advice in each and every step of my work. My special thanks to Mr. Yugal Kishore Mohanta, Mrs. Neelam Amit Kungwani, Jaya Chakraborty, Supriya Kumari, Rituraj (Phd Scholars) Department of Life Science, National Institute of Technology, Rourkela for their timely help, good wishes and moral support during my dissertation.

I would also like to thank all my classmates and my lab mates Pabitra Bhagawati, Manorama Mohanty, and Renu Sharma for their help and support during my work.

Last but not the least I express my heartiest devotion to my beloved parents for their ethical and moral support, love and blessings which helped in successful completion of my dissertation.

I bow my head before the Almighty for his blessings on me.

Declaration

I hereby declare that the thesis entitled **"Genetic Regulation of Allolysis In Response to Antibiotic Stress in *Streptococcus pneumoniae*"** submitted to Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the requirements for the degree of master of science in life science is an original piece of research work done by me under the guidance of Dr. Surajit Das, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. No part of this work has been done by any other research person and has not been submitted for any other purpose.

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List of Symbols and Abbreviations Used

-	Negative
+	Positive
µl	microlitre
BHI	Brain Heart Infusion Broth
C	Centigrade
CFU	Colony Forming Unit
gm	Gram
H	Hour
LB	Luria broth
MHB	Muller Hinton Broth
Min	Minute
Ml	Millilitre
NB	Nutrient Broth
No.	Number
^{o}	Degree
rpm	Revolution per minute
TSA	Tryptic soy agar
TSB	Tryptic soy broth

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Streptococcus pneumoniae is one of the major human pathogens that cause diseases like pneumonia, meningitis, otitis, acute conjunctivitis etc. Day by day the organism is acquiring resistance against many common antibiotics. One of the principal processes behind this is natural genetic transformation in the organism achieved by competence. Competence is a stress dependant mechanism in the bacteria. Another phenomenon that is induced by competence termed as allolysis is a peculiar process of the organism. In this process a group of cells which attain competence kill a group of cells that are non-competent. The mechanism involves bacteriocins, lysozymes and autolysins. This is believed to increase the efficiency of genetic transformation. But later it was assumed to have some other purposes like host pathogen interaction, pathogenecity and virulence and evolution of the species. In this study we tried to find out the effect of sub-lethal dose of antibiotic on allolysis. Chloramphenicol, the antibiotic against which the organism has developed resistance in the course of evolution was taken. The increase in eight fold in the expression of gene *cbpD* under stress condition showed that along with competence, allolysis also acts as a general stress response mechanism in the organism.

Key Words: Competence, allolysis, bacteriocins, virulence, stress response mechanism

1. INTRODUCTION

According to *The Penguin Dictionary of Biology*, a short clear definition of life can be made as "Life: Complex physico-chemical systems whose two main peculiarities are (1) storage and replication of molecular information in the form of nucleic acid, and (2) the presence of (or in viruses perhaps merely the potential for) enzyme catalysis."

To maintain life, starting from Archeabacteria to the most evolved and complex form of eukaryotes every organism carries out many processes in their cells. Microbial world which is invisible to the naked human eye also has certain complex processes that are even more complex than eukaryotic system. Especially pathogenic microbes have certain more interesting and special characteristic features as they need to maintain a stable host pathogen relationship. The prokaryotes like microbes are said to have an unorganized nuclear material without histone proteins. Still they have more proficient genetic material as there is the presence of only coding part in their genome. How the primary form of nucleus regulates such complex processes in microorganisms creates enormous interest to study the mechanism behind such physiological pathways.

1.1 *Streptococcus pneumoniae*: General Characteristics and Pathogenecity

Streptococcus pneumoniae is a Gram-positive cocci that generally stay in the “diplococcus” form i.e. two oval cells clumped to each other or may occur in a chain (Fig 1). It is included under class Bacilli, family Streptococcaceae and genus *Streptococcus*. It is highly pathogenic. One of the interesting characters shown is “Alpha Haemolysis” on blood agar plate. It also shows sensitivity towards a chemical “Optochin”. It is best known for its vital contribution in Griffith’s transformation to prove that “DNA is the genetic material”.

It is one of the major pathogenic bacterium that causes diseases like pneumonia, meningitis, otitis, acute conjunctivitis. Pneumonia is responsible for high mortality rate in developing countries (Greenwood 1999). The major proteins that are responsible for pathogenecity of

Streptococcus pneumoniae include thiol-activated toxin pneumolysin (Ply) and pneumococcal surface protein A (PspA) (Paton *et. al.*, 1993; Paton 1996; Briles *et. al.*, 1998).

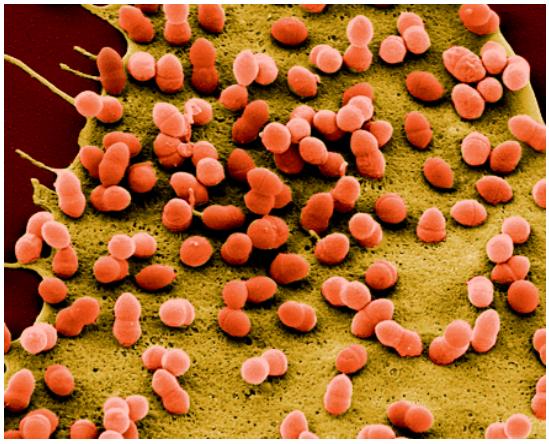

Fig 1: Morphology of *Streptococcus pneumoniae*

1.2 Antibiogram of *Streptococcus pneumoniae*

Streptococcus pneumoniae is generally susceptible to beta-lactum group of antibiotics. But gradually they attained increased Minimum Inhibitory Concentration (MIC) against various antibiotics such as tetracycline, erythromycin, chloramphenicol, clindamycin, streptomycin etc. (Klugman and Koornhof 1998). In India, *S. pneumoniae* is responsible for high mortality rate and it is reported to be acquiring resistance against most of the common antibiotics (Vashishtha 2000; Song *et al.*, 2004; Lalitha *et al.*, 2004). The bacterium is capable to take up foreign DNA through a transient state called competence that accounts for natural genetic transformation (Claverys *et al*, 2002). The ability of the organism to take up DNA from its environment is responsible for significant increase in its multidrug resistant property.

1.3 Competence in *Streptococcus pneumoniae*

Competence is a phenomenon shown by many bacterial cells in which a cell is capable of taking extracellular DNA. In *Streptococcus pneumoniae* competence is seen to be a population density dependant mechanism which is induced by Competence stimulating Pheromone peptide or CSP. Around 1-10 ng/ml concentration of CSP is able to induce competence in a liquid culture of *Streptococcus pneumoniae* which is found to be present in the extracellular culture when 10^7 no of cells/ml are present in the culture media. The CSP is known to be working via quorum sensing

mechanism. This is basically a population stress response mechanism in the organism (Steinmoen *et al.,* 2003).

1.3.1 Genetics of Competence

In the organism the competence development and regulation involves a very complex genetic mechanism. The *com* regulon is the principal operator of the process. It consists of 105–124 genes those respond to CSP (Peterson *et al.,* 2004). Among them only 22 genes (classified into 8 early and 14 late) genes actively take part in competence and the rest around 70 genes have no role in this process. Therefore they are supposed to have some other metabolic functions (Guiral *et al.,* 2005; Prozorov and Danilenko, 2011).

The microarray DNA mapping of CSP-responsive genes in *Streptococcus pneumoniae* revealed that the early gene encodes for seven important proteins required to start the cascatte. These are the ComAB : secretion apparatus of CSP , ComC : the precursor of CSP, ComDE : the two-component regulatory system where ComD : a histidine kinase and ComE : cognate response regulator of ComD, ComX (encoded by two identical genes, *comX1* and *comX2*) : the alternative sigma factor and ComW : recently discovered positive regulator of competence (Fig2). The ComX regulon encodes for late genes that secrete proteins involved in DNA uptake, binding and recombination process. (Steinmoen *et al.,* 2002; Peterson *et al.,* 2004; Guiral *et al.,* 2005; Kausmally *et al.,* 2005; Prozorov and Danilenko, 2011).

1.4 Allolysis

When competence is induced in a population of *Streptococcus pneumoniae,* a sub-fraction of the cell population are killed. The death of the cells is caused by some differentiated cells of the same population (Steinmoen *et al.,* 2002). Since cells of the same strain are involved in killing the process is termed as allolysis or fratricide i.e. killing of siblings (Prozorov and Danilenko, 2011).

This process is initiated after competence induction. Some of the cells become competent and they induce lysis of the non-competent cells present in the population. The lysed cells release their contents like DNA and proteins into the culture medium. These DNA and proteins are taken up by the competent cells. Therefore the process seems to be a predatory mechanism in which

the competent cells get benefited by DNA uptake and virulence protein like Ply at the expense of some of the cells in the population (Steinmoen *et al.*, 2002; Steinmoen *et al.*, 2003; Guiral *et al.*, 2005).

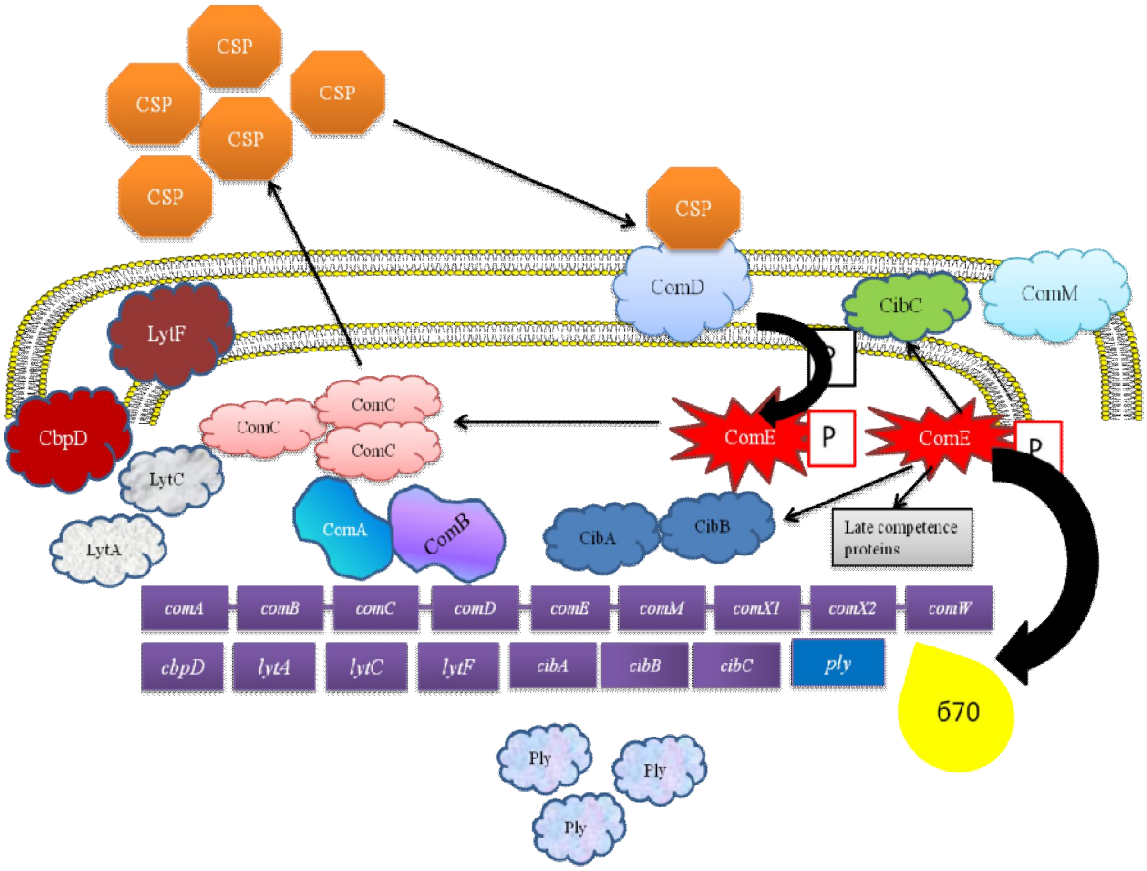

Fig 2: Genetic Regulation of Competence; ComC : codes precursor of CSP, ComAB: transporter protein that takes CSP to extracellular medium, ComD: Histidine kinase, a membrane receptor of CSP and phosphorylates ComE, ComE: cognate response receptor of ComD, ComM: immunity factor, ComW: positive regulator of competence, ComX: codes for alternative sigma factor which activates transcription of late competent genes, CbpD: takes part in lysis of non-competent cells, LytA: autolysin, LytC : lysozyme, LytF: functional analogue of CbpD, CibAB: bacterocin A and B for lysing the cells, CibC: immunity to cibAB, Ply: virulence factor.

Various peptides are responsible for the lysis of the cells. All together six genes play major roles in this process among which one belongs to *com* regulon (Guiral *et al.*, 2005). Autolysins like LytA, LytC and a murine hydrolase choline binding protein D (CbpD) play key roles in hydrolyzing the non-competent cells (Kausmally *et al.*, 2005, Prozorov and Danilenko, 2011). The two peptide bacteriocin competence induced bacteriocin A and B i.e. CibA and CibB

induced by the *com* operon are known to trigger the lysis of cells. This system was found when a LytA LytC double mutant was unable to reduce cell lysis in the population thus indicating role of additional elements in the mechanism (Guiral *et al.*, 2005).

The competent cells become resistant to lysis with the help of some immunity factors. These include proteins like ComM found in the cell membrane of competent cell and another one is CibC (Guiral *et al.*, 2005). These factors are absent in non-competent cells whereas the lysins involved in the process like LytA and LytC can either be contributed by competent cells or non-competent cells. The cell lysis is brought about by cell to cell contact of both competent and non-competent cells. The competent cells release lysins that anchor to their cell walls through choline binding domains and cause lysis of the target cells. (Fig 3) The rate of cell lysis increases with the increasing ratio of competent to non-competent cells in the culture medium (Steinmoen *et al.*, 2003; Guiral *et al.*, 2005).

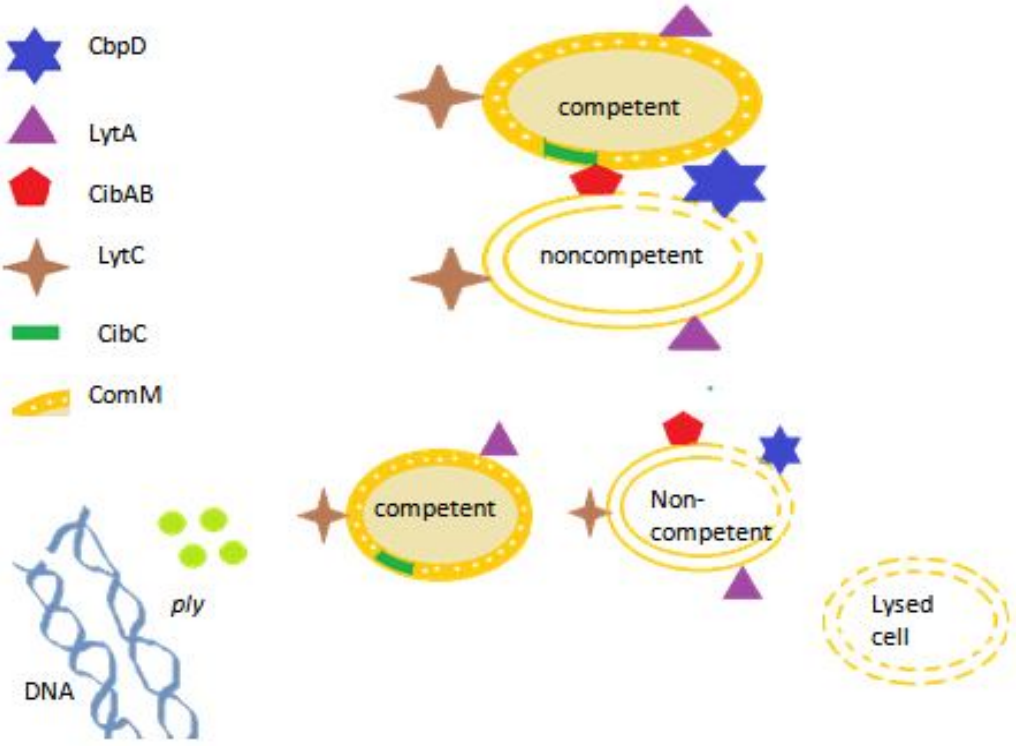

Fig. 3: Various proteins involved in the process of allolysis. Competent and non-competent cells come in contact, CibAB , CbpD: present in competent cell only and help in lysis of non-competent cell; ComM, CibC: immunity factor of competent cell; LytA, LytC : provided by either of the cells; DNA and ply released from non-competent cells is taken up by competent cell (Prozorov and Danilenko, 2011)

1.5 Bacteriocins and Fraticins

Bacteriocins may be defined as proteinacious toxins secreted by bacterial cells that are capable of lysing cells of same strain or relatively closer group of bacterial species. The class III bacteriocins involve bacteriolysins to which the lysins playing important role in allolysis can be added. Since the enzymes like lytA, lytC and a murine hydrolase choline binding protein D (cbpD) secreted by the competent cells carry out allolysis or fratricide, they are also termed as fraticins (Berg *et al.*, 2012).

1.6 Significance of Allolysis

Competence development occurs in the liquid culture of *Streptococcus pneumoniae* by a peptide pheromone Competence stimulating pheromone or CSP. This has been reported to be a population density dependant quorum sensing mechanism. But recent studies have revealed that CSP is not only an indicator of population stress rather it can act as a general stress alarm for any kind of stress in the culture medium like nutrition, pH or antibiotic stress (Claverys *et al.*, 2006).

Competence serves as a mode for natural genetic transformation in the species as it allows the cells to take up extracellular DNA. So competence induced allolysis might have role in increasing the efficiency of the genetic exchange among *Streptococcal* sp. But the process might have other significance as this continues even after the competence period is over (Moscoso and Claverys, 2004).

One of the most possible causes of allolysis may be the coordination of virulence in the organism as it leads to release of the virulence protein pneumolysin or Ply that requires disruption of the cell (Guiral *et al.*, 2005). It could be considered as a programmed cell death which shows a clear competition among the strains (Engelberg-Kulka and Hazan, 2003). It may play significant roles in host-pathogen relationship (Gilmore and Haas, 2005).

The antibiotic stress has been reported to induce competence in *Streptococcus pneumoniae.* Not all antibiotic can induce the process but most of the DNA damaging antibiotics are capable of inducing competence except the beta lactam group. In this study, we have tried to establish the relationship among sub-lethal antibiotic stress and induction of allolysis in case of *Streptococcus pneumonia* (MTCC655). We have also studied the expression pattern of various genes involved

in allolysis under stress condition to confirm the phenomenon of stress induced allolysis along with competence. Based on which, the strategies may be prepared for antibiotherapy against the target pathogen.

2. REVIEW OF LITERATURE

2.1 Systematic position of the *Streptococcus pneumoniae*

In biological world the organism has been placed as follows.

Domain: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Lactobacillales

Family: Streptococcaceae

Genus: *Streptococcus*

Species: *S. pneumoniae*

2.2 General Features

George Sternberg and Louis Pasteur for the first time isolated Pneumococcus, the organism as the causative agent of pneumonia in the year 1881. In 1886 Fraenkel discovers *Streptococcus pneumoniae,* a cause of pneumonia. Since 1920 the organism was termed *Diplococcus pneumoniae* but later renamed as *Streptococcus pneumoniae* in 1974 because of its growth in chains in liquid media.

It is a member of high G+C Gram positive aerobic oral streptococci group often found as two cells attached to each other forming a lancet shape. It is a common human flora that inhabits the mouth, oropharynx and nasopharynx of upper respiratory tract. But it develops pathogenecity under suitable conditions. The organism shows alpha haemolysis pattern when plated on blood agar by producing hydrogen peroxoide which oxidizes haemoglobin to methemoglobin. This is characterized by a change in colour of the blood agar from red to green. Hence it is also called as partial or incomplete hemolysis. *S. pneumoniae* is distinguished by two important tests i.e. bile solubility test and optochin sensitivity test. Bile degrades the cell wall by lysing the cells and the chemical optochin or ethylhydrocupreine also kills the organism. There exist more than 90

serotypes of this organism which are classified on the basis of virulence, prevalence and drug resistant properties.

2.3 Genome Composition of *Streptococcus pneumoniae*

The genome of *S. pneumoniae* cosists of a closed, circular naked DNA containing around 2.0 and 2.1 million basepairs which may vary among strains. 1553 no of core genes, out of which 154 genes are responsible for virulence and 176 genes maintaining the non-invasive phenotype have been identified (T and Opal, 2009).

2.4 Pathogenecity and Host Pathogen Interaction

On the basis of causing diseases *S. pneumoniae* may be two types i.e. virulent and avirulent. Capsules present around bacteria containing polysachharides contribute to its disease causing property or virulence. It is the leading agent of causing pneumonia. In addition to this other major diseases caused by *S. pneumoniae* include acute sinusitis, otitis media, meningitis, bacteremia, sepsis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis and brain abscess etc. mainly causes diseases in old age groups and in children under age of five years (Bogaert *et al*., 2004). It also shows interactions with *Haemophilus influenza* when cocultured. The growth of *Haemophilus influenza* is suppressed in presesnce of *S. pneumoniae*. These bacteria secrete a coagulase enzyme that precipitates a fibrin coat around them, shielding them from phagocytic cells (Willey *et. al*., 2011).

2.5 Antibiotic susceptibility of *Streptococcus pneumoniae*

Since 19th century *Streptococcus pneumoniae* has been reported to acquire multi drug resistance bacteria having resistance against antibiotics like beta lactam group, tetracycline, erythromycin, sulfa drugs and chloramphenicol, to which previously they were sensitive. Earlier an increase in value of MIC was also reported against antibiotics like tetracycline, erythromycin, chloramphenicol, clindamycin, streptomycin, and, for some isolates, rifampin (Klugman and Koornhof 1998).

One of the principal mechanisms behind acquiring multi drug resistance is natural genetic transformation acquired through competence. By this process the organism acquires

heterogenous DNA integrated into its genome via recombination and helps in altering the target site of the antibiotics and creating more no of serotypes of the infectious organism (Klugman and Koornhof 1988; Tomasz 1997).

2.6 Genetic Regulation of Competence

Competence in *Streptococcus pneumoniae* is a well characterized phenomenon. It is a cell density dependent quorum sensing process mediated through a signalling molecule known as competence stimulating petptide pheromone (CSP). The whole process is controlled by *com* operon consisting of *comABCDE* genes. The extracellular concentration of CSP at 1-10 ng/ml which corresponds to 10^7 no of cells/ml is enough to trigger competence in the culture of the *Streptococci* cells. The role of various genes involved in competence has been described.

***comA* and *comB* :** Encode two proteins ComA and ComB that is the secretion apparatus of the CSP and a proteolytic ATP Binding Cassette (ABC) transporter that takes part in processing pre-CSP (Havarstein *et al.,* 1995) and transport of mature CSP across cytoplasmic membrane into extracellular medium. In ComA both Cys17 and His96 take part in catalysis (Ishii *et al*. 2006).

comC: It codes the precursor of CSP which then undergoes proteolytic cleavage by ATP Binding Cassette (ABC) transporter and is transported across the cytoplasmic membrane as matured peptide. There are two alleles of the gene *comC1* and *comC2* that codes for CSP1 and CSP2 which are serotype specific. The 17 amino acid peptide CSP is formed by the proteolytic cleavage of the 24-aa GG-leader (Glycine) at N termini of the pre-CSP. The accumulated CSP in the medium leads to stimulation of a two-component regulatory system *comCD* (Havarstein *et al,* 1995; Pestova, *et al*, 1995).

comD: It codes for a membrane localized histidine kinase which acts as the receptor for CSP. It gets autophosphorylated upon binding to the pheromone and transphosphorylates its cognate response regulator ComE (Claverys and Havarstein, 2002).

comE: It is activated by transphosphorylation process due to addition of a phosphate group to its conserved receptor module by ComD and in turn helps in activation of early genes including *comABCDE* that helps in extracellular accumulation of CSP. It also activates *comX* gene that activates transcription of late genes responsible for DNA uptake, binding and recombination. In

phosphorylated form it also serves as a promoter sequence for genes upragulated during development of competence state (Lee and Morrison, 1999).

comX: It encodes an alternative sigma factor which activates the transcription of late competence genes. Sigma factor along with RNA polymerase recognizes the consensus promoter sequence of late genes called a cin-box (TACGAATA) and activates their transcription. The 14 essential genes coding for proteins taking part in DNA uptake, binding and recombination are part of com regulon. This also activates the transcription of genes like *cbpD, lytA* and *cibAB that* are absolutely essential for allolysis (Campbell *et al.,* 1998; Lee and Morrison, 1999).

comM: It belongs to the block of early competent genes. It encodes the protein ComM that gives immunity to the competent cell against lysis action of CbpD protein. Therefore it has been reported to be expressed 5 min before expression of *cbpD*. The mechanism of immunity is still unknown (Eldholm *et al.*, 2010).

comW: A recently discovered early competent gene. This is known to promote the competence and is a positive regulator of the *com* operon. It is also activated by CSP. It is responsible for stabilization and activation of ComX (Luo *et al.*, 2004).

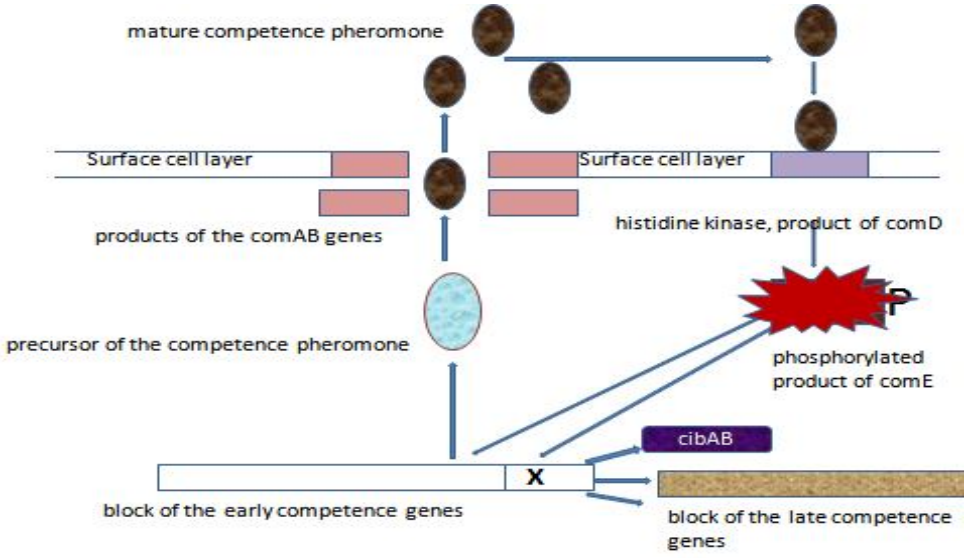

Fig 4: Regulation of competence in *S. Pneumonia*e. Precursor of CSP is transported by product of ComAB gene and becomes mature pheromone; the membrane bound histidine kinase ComD is the receptor of CSP and it transphosphorylates ComE. Then ComE activates block of early competence genes i.e. ComCDE and ComX. ComX codes for bacteriocins cibAB and block of late competent genes.

cibA and cibB: Belong to the late competent genes and are named as Competence induced bacteriocin. The genes consist of small *orfs, orf62-orf51* and have the potential to encode bacteriocins with double glycine (GG) leaders. The mature peptides consist of 36 and 28 amino acids respectively. Both the peptides CibA and CibB act together and are included under family of two peptide bacteriocins (Guiral *et al.,* 2005).

cibC: Consists of an ORF with a potential to code for a 65 long amino acids residue. It produces an immunity factor that protects the cell against lysis by CibAB. It is seen to be present in *cibABC* as a transcript along with *cibA* and *cibB* (Guiral *et al.,* 2005).

cbpD: It is part of the ComX regulon and is expressed only during competence. It is secreted by a Sec apparatus. It is a murine hydrolase. It consisists of 448 amino acids sequence with molecular weight of 50kDa and has three distinct domains along with a signal sequence at the N-terminal end. A 150 amino acid long CHAP (cysteine, histidine dependent amidohydrolase/peptidase) is present downstream to signal sequence followed by two SH3 domains and a C-terminal choline binding domain (CBD) with four repeats. CBDs consist of a variable number of tandem repeat units, each of which has 20–21 amino acids. It remain attached to phosphorylcholine moieties linked with teichoic and lipoteichoic acid in the cell wall of *Streptococcus pneumoniae* through non covalent interactions (Eldholm *et al.,* 2010).

lytF: This is also a competence induced mureine hydrolase coding gene that belongs to the late competent genes. Its product LytF shares similarity with CHAP domain of CbpD and it serves as a functional analogue of CbpD. It is expressed only in competent cells that lack CbpD (Berg *et al.,* 2012)

lytA*:* Codes for the major autolysin of *S. pneumoniae* which is an acetylmuramyl-L-alanine amidase. Gene encoding the enzyme also belongs to ComX regulon. It causes the autolysis of *S. pneumoniae* at stationary phase for releasing the cytolytic toxin pneumolysin (Ply) thus coordinating the virulence of the organism. LytA has a molecular weight of 36kDa and has two domains. It can decompose the *Streptococcal* cell wall by targeting the choline residues of teichoic and teichuronic acid. It is reported to be expressed more during competence as one of its promoters is induced by competence (Mosser and Tomasz, 1970).

lytC: It codes for a lysozyme having molecular weight 55kDa and consists of two domains. It is known to have secondary role in lysis of cells by CbpD. No other biological role has been defined for this till now. It does not belong to competence inducible genes (Prozorov and Danilenko, 2011).

ply: It is the pneumolysin coding gene that contributes to the virulence of *S. pneumoniae.* It is synthesized and stored in the cytoplasm of the cell. It requires the lysis of the cell to come out. LytA plays a vital role in lysing the cell and releasing of the virulence factor into extracellular medium. This toxin causes hemolysis of the erythrocytes (Paton *et al.,* 1993).

2.7 Competence Induction

In a liquid culture of *S. pneumoniae* when the CSP reaches a critical concentration in the extracellular medium it stimulates competence. This is said to be a cell density dependant quorum sensing mechanism. The CSP binds to its receptor which is a membrane bound histidine kinase, ComD and activates it. The activated ComD transphosphorylates ComE. The phosphorylated ComE activates transcription of *comC* and leads to more production and accumulation of CSP and thus stimulates the competence process. The activated ComE also activates the production of an alternative sigma factor $б^{70}$ which is a product of *comX*. This ComX activates the transcription of late competent genes that code proteins responsible for uptake, binding and recombination of extracellular DNA. The competence provides DNA uptake to serve as material for genetic exchange, DNA repair and to acquire toxin and food.

Previously it was assumed that only cell density activates the competence induction. Later it was found that not only population stress rather any kind of stress like antibiotic, pH can also induce competence thus indicating that CSP acts as a general stress response pheromone and competence is a general stress response mechanism (Claverys *et al.*,2006).

2.7.1 pH change and competence

When the culture of *Streptococcus* is grown in a more alkaline medium, it has been seen that competence develops after a particular time independent of the cell density of the culture. This suggests that competence development does not depend on passive accumulation of CSP rather

competence can develop by alternation expression of *comCDE* in response to environmental stress (Claverys *et al.*,2006).

2.7.2 Antibiotic stress and competence

Streptococcus pneumoniae, which lacks a SOS like repair system uses competence as a strategy to repair the DNA damage caused by antibiotic or DNA damaging agent. It has been reported that in response to Mitomycin C, a DNA damaging agent caused induction of competence in *Streptococcus pneumoniae.* Several other antibiotics were also reported to induce competence. These include protein synthesis inhibitors like kanamycin and streptomycin induced competence but erythromycin and tetracycline could not. Similarly fluoroquinolones like norfloxacin, levofloxacin, and moxifloxacin which act on type II topoisomerases, DNA gyrase, and topoisomerase IV could induce competence. DNA gyrase inhibitor novobiocin, the RNA polymerase inhibitor rifampicin, the glycopeptide antibiotic vancomycin or the β-lactams ampicillin and the third-generation cephalosporin, cefotaxime were unable to trigger competence (Pruhomme *et al.*, 2006).

2.8 Competence and Allolysis

Till date the phenomenon allolysis have been well studied in two Gram positive bacteria i.e. *Streptococcus pneumoniae* and *Bacillus subtilis*. It is also termed as fratricide. In *B. subtilis* the allolysis follows the competence at the onset of sporulation and in *S. pneumoniae* it follows competence induced in response to stress. The process involves lyse or killing of a sub-fraction of the cells by group of genetically similar cells of the same population in the same culture medium.

2.8.1 Mechanism of Allolysis in *Streptococcus pneumoniae*

The lysis of the non-competent cells is brought about by bacteriocins. The bacteriocins are produced by competence inducible genes. These include CibA, CibB, LytA and CbpD. The cells that become competent express CibA, CibB, CbpD and immunity factors ComM and CibC. LytC is not the product of competence inducible gene and it only has supplementary role in CbpD mediated cell lysis. Both LytA and LtyC are expressed by competent as well as non-competent cells.

The lysing of the non-competent cell is brought about by cell to cell communication or cell to cell contact. The bacteriocins CibAB trigger the lysis of the cell by inserting into the membrane of the sensitive cells and by lowering their cellular energy. This facilities further lysis of the cell by CbpD, LytA and LytC. The CbpD binds to the peptidoglycon with the help of SH3 domain and then the CbpD is directed towards the division zone by choline-binding domain. This double binding of CbpD with teichoic acid and peptidoglycan via its CBD and SH3b domains respectively leads to lysis of the target cells. The CbpD accumulates in the septal region and lyses the cell by rupturing it at the division zone (Dagkessamanskaia *et al*., 2004).

2.8.2 Biological significance of allolysis

Although the exact biological significance of allolysis is yet to be elucidated the most probable significance stated includes increase in the efficiency of horizontal gene transfer by donating DNA that helps the organism to acquire new characters like antibiotic resistance. But the existence of this process even after the competent state is over suggests that genetic plasticity through horizontal gene transfer could not be the only purpose of this process (Moscoso *et al.,* 2004).

It constitutes an example of programmed cell death where the competent cells are benefited by taking up DNA and nutrients released by non-competent cells via a predatory mechanism as that of shown by the sporulating cells of *Bacillus subtilis.* This is also an important process that leads to release of the pneumolysin, the virulence factor of *S. pneumoniae* which requires lysis of the cells for its release.

The released products act as inflammatory compounds and the bacterial DNA causes septic shock and elicit the production of cytokine along with lymphocyte proliferation. This suggests that the process plays an important role in the coordination between competence and virulence in *S. pneumoniae*. It can also enhance the virulence of *S. pneumoniae* by provoking the release of complementary virulence factors from related *Streptococcal* strains. It also benefits the competent cells to acquire non-heritable variability from the pool of virulence factors.

Increase in DNA uptake contributes towards its multi-drug resistance property and provides evidence for evolution of the species. It might play important role in the host pathogen relationship of *S. pneumoniae* as the ply released stimulates the Toll like receptor 2 that in turn

activates the host innate immune system against the pathogen Thus the mechanism might actively alert the host defence system and indicate its presence that leads to stabilization of the pathogen inside host with prolonged period of colonization or it may cause severe infection by secreting the virulence factors (Gilmore and Haas, 2005).

The allolysis may have many crucial roles in biology of *S. pneumoniae* and therefore it can be assumed to be a general property of the species (Guiral *et al.,* 2005).

3. OBJECTIVES

Based upon the previous results and the literature surveyed the current work was planned with the following objectives:

1- Characterization of the *S. pneumoniae* strain MTCC655

2- Study of induction of allolysis in *S. pneumoniae* by sub-lethal antibiotic stress

3- To study the genetic mechanism of allolysis of the isolate by amplifying *lytA*, *lytC* and *cbpD* gene

4- To study the changing pattern of *cbpD* expression in presence and absence of antibiotic stress

4. MATERIALS AND METHODS

4.1 Designing of primers for allolysis associated genes

Gene sequences were obtained from National Center for Biotechnology Information (NCBI) database. Then primers were designed for the genes *cibA*, *cibB*, *cbpD* and *ply*.

Primers were designed using NETPRIMER software.

Netprimer is software used to design and analyze the parameters of designed primer sequences using the following steps.

1. To start with Netprimer the following link is browsed.

   http://www.premierbiosoft.com/netprimer/index.html

2. In the webpage log in is required. After logging in the following webpage appears on the screen

3. Launch Netprimer icon is clicked.

4. Manually sequence of around 20-22bp is selected from the whole genome sequence and sequence was given to netprimer software in 5'-3' orientation.

5. Netprimer primer designing and analysis tool appears.

6. The set of default values values can be changed according to requirements.

7. The Analyze tab is clicked.

8. In the following screen all analyzed parameters including Tm, GC content, hairpin, dimmers, cross dimmers etc are shown.

9. Hiarpin, Dimer, Palindrome, repeat and Run tabs are clicked for the presence of these parameters in the sequence.

10. Same steps are done for reverse primer sequence. 20-22bp are selected from such a region so that an amplicon size of 1kb is obtained.

11. Oligotype option is changed to antisense.

12. The difference between Tm for both the primers is checked for not more than 5^{o}C.

13. The rating indicates quality or efficiency of primer to give desired amplicon.

4.2 Subculture of MTCC culture of *Streptococcus pneumoniae* MTCC655

The lyophilized culture of *Streptococcus pneumoniae* MTCC655 was re-suspended in 5 ml of Tryptic Soy Broth (TSB) (pancreatic digest of casein 17g/l, papaic digest of soyabean meal 3g/l, sodium chloride 5g/l, dipotassium hydrogen phosphate 2.5g/l, dextrose/glucose 2.5g/l). Culture was incubated in aerobic condition at 37^{o}C.

4.3 Gram's staining

A thin smear of overnight grown culture was prepared on a clean and dry slide. It was air dried and heat fixed. The primary stain crystal violet was applied to the smear for 1 min. The slide was then washed gently with tap water. Gram's iodine was added then as mordant for 1 min. Again the slide was gently washed with tap water followed by the addition of Gram's de colourizer until the disappearance of violet colour from the slide. Again the slide was rinsed with tap water and counter stained with saffranin for 1 min. Slide was washed with tap water and air dried. Then it was observed under oil immersion microscope (1000X Olympus).

4.4 Blood Hemolysis Test

An isolated pure colony of was taken with the help of a sterile inoculum loop grown on Tryptic Soy Agar (TSA i.e. TSB with 1.5% agar) plate and it was streaked on a Blood Agar Plate (TSA supplemented with 5% sheep blood). Then this plate was incubated in aerobic condition at 37^{o}C for 12 hr and then observed for the pattern of hemolysis.

4.5 Bile Solubility Test

Bile solubility test was performed following "Center for disease control and prevention: Department of Health and Human services, USA" protocol.

1.0 ml saline (0.8% NaCl dissolved in sterile miliQ water) solution was prepared and suspension was divided into 2 tubes with approximately 0.5 ml in each tube. In one tube a suspension of *Streptococcus pneumoniae* MTCC655 cells was prepared from overnight grown culture on an agar plate and in other tube and in another tube a suspension of *Pseudomonas sp.* was prepared. Turbidity equal to that of 1.0 to 2.0 McFarland density standards was used. 0.5 ml of 2% sodium deoxycholate (bile salts) was added to each tube and was mixed by vigorous shaking. The tubes were incubated at 37^{o}C for up to 2 hr and then analyzed.

4.6 Biochemical characterization (Sugar Utilization Test)

Biochemical characterization was done by using Hi-Media KB009 Part B kit containing 12 carbohaydrates in the order inulin, sodium gluconate, glycerol, salicin, glucosamine, dulcitol, inositol, sorbitol, mannitol, adonitol, α methyl D glucoside and ribose. 50 µl of overnight grown culture was added to each well and incubated at 37^{o}C for 12 hr and examined.

4.7 Growth Curve of *Streptococcus pneumoniae* MTCC655

To study the growth pattern of *Streptococcus pneumoniae* MTCC655 it was grown in 200ml of Nutrient Broth (NB) media (Peptic digest of animal tissue 5g/l, sodium chloride 5g/l, beef extract 1.5g/l, yeast extract 1.5g/l) by adding 2ml of overnight grown culture in Brain Heart Infusion Broth or BHI (calf brain 200g/l, beef heart 250g/l, protease peptone 10g/l, dextrose 2g/l, sodium chloride 5g/l, disodium phosphate 2.5g/l) and incubated at 37^{o}C. Then 200 µl of culture was taken in a microtitre plate starting from 0 hr of incubation to 10^{th} hr of incubation at an interval of 1hr. The Optical density (OD) was measured at 595nm wavelength in ELISA plate reader and growth curve was plotted ((Willey *et. al.*, 2011).

4.8 Minimum Inhibitory Concentration of Antibiotic

Antibiotic stock solution was prepared by adding 10 mg of Chloramphenicol in 1ml of Dimethylsulphoxide (DMSO). The MIC is the lowest concentration of the antibiotic which is

inhibitory to the development of visible growth. For this a 96 well plate was taken and labelled from 1 to 12 and no.11 and 12 were taken as positive control and negative controls respectively. 150µl of Muller Hinton Broth medium (Beef infusion 300g/l, Cassein acid hydrosylate 17.5g/l, starch 1.5g/l) was added in all wells and then 150µl antibiotic (stock-10 mg/ml) was added to 1^{st} well and to positive control. Antibiotic was mixed properly by repeated pipetting. 150µl of properly mixed broth medium from 1^{st} well was transferred into the 2nd well and by this antibiotic was serially diluted upto 10^{th} well. To each of the wells except the negative control, 10µl of properly diluted 12hrs culture of Streptococcus *pneumoniae* MTCC655 organism was added. Then the plate was incubated at 37^{o}C for 12 hr and was analyzed by visualizing the turbidity and measuring the OD in ELISA plate reader (CLSI 2006).

4.9 Antibiotic Sensitivity Test

Antibiotic susceptibility test was performed by disk diffusion method. Fresh overnight grown culture of *Streptococcus pneumoniae* MTCC655 was used for the detection of antibiotic susceptibility of the organism (Bauer *et al.*, 1966).

A sterile nontoxic cotton swab was dipped into the liquid broth culture and by pressing against the wall of the tube excess liquid was removed from the soaked swab. The plates were streaked with the cotton swab three times rotating the angle at 60^{o} in each rotation. The inoculum was allowed to dry for some time on each plate. With the help of a sterile needle the discs were deposited on the plates at a considerable distance from each other. Then plates were incubated at 37^{o}C for 12 hr and the examined. The zones of inhibition were determined and sensitivity for each antibiotic was interpreted with help of Himedia Private Limited user guide.

4.10 In vitro Allolysis Testing

A. By Viable Cell Count

This is a novel approach to study the effect of allolysis on viable cell concentration under normal growth condition and under sub lethal antibiotic stress condition in *Streptococcus pneumoniae* MTCC655.

The culture of *Streptococcus pneumoniae* MTCC655 was grown in BHI medium at 37^{o}C in around 160rpm overnight. Plates containing Nutrient agar or NA ((Peptic digest of animal tissue 5g/l, sodium chloride 5g/l, beef extract 1.5g/l, yeast extract 1.5g/l, Agar 15g/l) were prepared. Then 2 flasks containing 200ml of Luria Broth (LB) (Cassein acid hydrosylate 10g/l, yeast extract 5g/l, sodium chloride 5g/l) were prepared. In one flask 320µl of antibiotic solution (stock solution 10mg/ml) was added after discarding that volume of media. 1ml of overnight grown culture was transferred to each flask. Immediately after the transfer 1ml of solutions were taken from each flask to 1.5ml micro centrifuge tubes. Then cultures were serially diluted and spreading was done on NA plates. The plates were incubated at 37^{o}C in around 160rpm for 12 hr and then colonies on each plate were counted. This process was continued up to 9th hr of incubation at an interval of 2 hrs. Along with this process each time 200µl of cultures were transferred to 96 well plate and absorbance was recorded in ELISA plate reader at 595nm.

B. By Quantitation of Extracellular DNA

Two tubes with 5ml of BHI were prepared. In one tube sublethal dose of chloramphenicol antibiotic was added. Then overnight grown culture was added to both the tubes and incubated at 37^{o}C in 160rpm. Before incubation 1ml of culture from each tube were transferred into 1.5ml micro centrifuge tubes. Both the tubes were centrifuged at 5000rpm for 5 min and the supernatant were transferred to fresh tubes. Then it was examined to detect presence of DNA in Nanodrop. The process was repeated till 10th hr of incubation at an interval of 1 hr.

To observe the hemolysis pattern 1µl of culture from both the tubes were put on Blood Agar Plates till 10th hr of incubation at an interval of 1 hr and plates were incubated at 37^{o}C for 24 hr and then analyzed.

4.11 Amplification of *cibA, cibB, cbpD* and *ply* genes responsible for allolysis at various conditions

Four different conditions were chosen to examine the amplification of the genes involved in allolysis. The cultures were prepared in BHI and incubated. DNA was isolated by cell lysate preparation method. Cultures were grown in presence and in absence of antibiotic. DNA isolation was done at four points of incubation which include culture containing antibiotic and

culture without antibiotic after 3 hr of incubation and also culture with and without antibiotic after overnight period.

I. **Preparation of cell lysate:** 300µl of culture was taken in 1.5ml micro centrifuge tube. It was centrifuged at 6000rpm for 5 min. Supernatant was discarded. The pellet was washed with 300 µl of sterile miliQ water. Again centrifuged at 6000rpm for 5 min. Supernatant was discarded and previous step was repeated. Then supernatant was discarded and the pellet was dissolved with 200µl of miliQ water. Then tube was placed in water bath at 100^{o}C for 15 min followed by immediate snap chill in ice for 5 min. The tube was then centrifuged at 8000rpm for 5 min. the supernatant was transferred to a fresh tube and stored at 4^{o}C for future use.

II. **Polymerase Chain Reaction:** For PCR reaction 25µl master mix was prepared. Reaction was carried out at initial denaturation of 95ºC for 5 min and followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 58ºC for 1 min and extension at 72ºC for 1 min, and final extension for 10min at 72^{o}C for and at holding temperature of 4 ºC. The PCR product was run in 1% agarose gel for interpretation of result.

4.12 Expression level of *cbpD* gene under various conditions by Real Time PCR

To study the expression level of *cbpD* gene essentially involved in allolysis quantitative real time PCR was performed by keeping 16S rRNA gene as an internal control. The expression pattern of cbpD gene was detected by analyzing sybrgreen concentration in the amplified product. Reaction condition includes initial denaturation of 95ºC for 2 min and followed by 40 cycles of denaturation at 95ºC for 15 sec, annealing at 58ºC for 15 sec and extension at 72ºC for 20 sec. From the data obtained analysis of gene expression was carried out by realplex software.

5. RESULT

5.1 Gene sequences obtained from NCBI

cibA

ATGACAAATTTTGACATTCTTGACAATCAATTTTTATCCTTATCTGAAAATGAATTATCAGATATTGAT
GGCGGTCTCGCTCCCTTGGTTATCTTTGGAGTAGCAGTATCTTGGAAGGCTATTGCAGGTGGAACAGC
ACTTATAGGTTCTGGTTTGGCAGCTGGTTATTTTTTAGGAGGAGATTAA

cibB

ATGATGAAAGATTTGAACAACTATCGTGAAATTTCTAATAAGGAATTGCAAGAAATCAAGGGTGGCTT
TGGTGTAGGTGTTGGTATCGCTTTATTTATGGCAGGTTATACCATTGGAAAAGACCTTCGTAAAAAGTT
TGGTAAATCATGCTAG

cbpD

ATGAAAATTTTACCGTTTATAGCAAGAGGAACAAGTTATTACTTGAAGATGTCAGTTAAAAAGCTTGT
TCCTTTTTTAGTAGTAGGATTGATGCTAGCAGCTGGTGATAGTGTCTATGCCTATTCCAGAGGAAATGG
ATCGATTGCGCGTGGGGATGATTATCCTGCTTATTATAAAAATGGGAGCCAGGAGATTGATCAGTGGC
GCATGTATTCTCGTCAGTGTACTTCTTTTGTAGCCTTTCGTTTGAGTAATGTCAATGGTTTTGAAATTCC
GGCAGCTTATGGAAATGCGAATGAATGGGGACATCGTGCTCGTCGGGAAGGTTATCGTGTAGATAATA
CACCGACGATTGGTTCCATTACTTGGTCTACTGCAGGAACTTATGGTCATGTTGCCTGGGTGTCAAATG
TAATGGGAGATCAGATTGAGATTGAGGAATATAACTATGGTTATACAGAATCCTATAATAAACGAATT
ATAAAAGCAAACACGATGACAGGATTTATTCATTTTAAAGATTTGGATGGTGGCAGTGTTGGGAATAG
TCAATCCTCAACTCAACAGGCGGAACTCATTATTTTTAAGACCAAGTCTGCTATTAAAACTGAACCCCT
AGTTAGTGCAACTGTGATTGATTACTATTATCCTGGAGAGAAGGTTCATTATGATCAAATTCTCGAAAA
AGACGGCTACAAGTGGTTGAGTTATACTGCCTATAATGGAAGCTATCGTTATGTTCAATTGGAGGCTG
TGAATAAAAATCCTCTAGGTAATTCTGTTCTTTCTTCAACAGGTGGAACTCATTATTTTAAGACCAAGT
CTGCTATCAAAACTGAACCCCTAGTTAGTGCAACTGTGATTGATTACTATTATCCTGGGGAGAAGGTTC
ATTATGATCAGATACTTGAAAAAGACGGCTACAAGTGGTTGAGTTATACGGCTTATAACGGAAGTCGT
CGCTATATACAGCTAGAGGGAGTGACTTCTTCACAAAATTATCAGAATCAATCAGGAAATATCTCTAG
CTATGGATCCAATAATAGTTCAACTGTCGGTTGGAAGAAAATAAATGGTAGTTGGTATCATTTCAAAT
CAAATGGTTCTAAATCAACAGGATATGGCTCGTGGTATTATCTGGGTAGTTCAGGGGCAATGAAAACA
GGCTGGTACCAGGTCTCTGGTAAGTGGTATTATTCTTACTCTTCAGGCGCCTTAGCTGTTAATACGACG
GTGGATGGCTACAGAGTAAACAGTGATGGAGAACGAGTATAG

ply

ATGGCAAATAAAGCAGTAAATGACTTTATACTAGCTATGAATTACGATAAAAAGAAACTCTTGACCCA
TCAGGGAGAAAGTATTGAAAATCGTTTCATCAAAGAGGGTAATCAGCTACCCGATGAGTTTGTTGTTA
TCGAAAGAAAGAAGCGGAGCTTGTCGACAAATACAAGTGATATTTCTGTAACAGCTACCAACGACAG
TCGCCTCTATCCTGGAGCACTTCTCGTAGTGGATGAGACCTTGTTAGAGAATAATCCCACTCTTCTTGC
GGTCGATCGTGCTCCGATGACTTATAGTATTGATTTGCCTGGTTTGGCAAGTAGCGATAGCTTTCTCCA
AGTGGAAGACCCCAGCAATTCAAGTGTTCGCGGAGCGGTAAACGATTTGTTGGCTAAGTGGCATCAAG
ATTATGGTCAGGTCAATAATGTCCCAGCTAGAATGCAGTATGAAAAAATCACGGCTCACAGCATGGAA
CAACTCAAGGTCAAGTTTGGTTCTGACTTTGAAAAGACAGGGAATTCTCTTGATATTGATTTTAACTCT
GTCCATTCAGGCGAAAAGCAGATTCAGATTGTTAATTTTAAGCAGATTTATTATACAGTCAGCGTAGA
TGCTGTTAAAAATCCAGGAGATGTGTTTCAAGATACTGTAACGGTAGAGGATTTAAAACAGAGAGGA

ATTTCTGCAGAGCGTCCTTTGGTCTATATTTCGAGTGTTGCTTATGGGCGCCAAGTCTATCTCAAGTTG
GAAACCACGAGTAAGAGTGATGAAGTAGAGGCTGCTTTTGAAGCTTTGATAAAAGGAGTCAAGGTAG
CTCCTCAGACAGAGTGGAAGCAGATTTTGGACAATACAGAAGTGAAGGCGGTTATTTTAGGGGGCGA
CCCAAGTTCGGGTGCCCGAGTTGTAACAGGCAAGGTGGACATGGTAGAGGACTTGATTCAAGAAGGC
AGTCGCTTTACAGCAGATCATCCAGGCTTGCCGATTTCCTATACAACTTCTTTTTTACGTGACAATGTA
GTTGCGACCTTTCAAAATAGTACAGACTATGTTGAGACTAAGGTTACAGCTTACAGAAACGGAGATTT
ACTGCTGGATCATAGTGGTGCCTATGTTGCCCAATATTATATTACTTGGGATGAATTATCCTATGATCA
TCAAGGTAAGGAAGTCTTGACTCCTAAGGCTTGGGACAGAAATGGGCAGGATTTGACGGCTCACTTTA
CCACTAGTATTCCTTTAAAAGGGAATGTTCGTAATCTCTCTGTCAAAATTAGAGAGTGTACCGGGCTTG
CCTGGGAATGGTGGCGTACGGTTTATGAAAAAACCGATTTGCCACTAGTGCGTAAGCGGACGATTTCT
ATTTGGGGAACAACTCTCTATCCTCAGGTAGAAGATAA GGTAGAAAATGACTAG

Based on the sequences obtained from NCBI and properties of an ideal primer, the set of forward and reverse primers were designed for PCR and RTPCR (Table 1).

Table 1: List of Primers used in PCR and RT PCR

GENE NAME	FORWARD PRIMER	REVERSE PRIMER	AMPLICON SIZE
cibA	GGTCTCGCTCCCTTGGTTAT	GCAGGTGGAACAGCACTTAT	
	Length 20bp	Length 20bp	
	T_m 58.26°C	T_m 55.05°C	71bp
	GC content 55%	GC content 50%	
CibB	GCAAGAAATCAAGGGTGGCT	CGCTTTATTTATGGCAGGTT	
	Length 20bp	Length 20bp	
	T_m 59.24°C	T_m 55.4°C	59bp
	GC content 50%	GC content 40%	
cbpD	GCTCGTCGGGAAGGTTATCG	GGATGGTGGCAGTGTTGGGA	
	Length 20bp	Length 20bp	
	T_m 62.03°C	T_m 63.41°C	232bp
	GC content 60%	GC content 60%	
Ply	GGCAAGTAGCGATAGCTTTCT	GTCAGGTCAATAATGTCCCA	
	Length 20bp	Length 20bp	
	T_m 55.59°C	T_m 52.57°C	118bp
	GC content 50%	GC content 45%	

5.2 Culture of *Streptococcus pneumoniae MTCC655* in TSB and TSA plate

The bacteria were grown in TSB and stored at 4°C till further use (Fig5a). Simultaneously, the bacterial colonies developed on TSA plates were small rounded and white in colour (Fig5b).

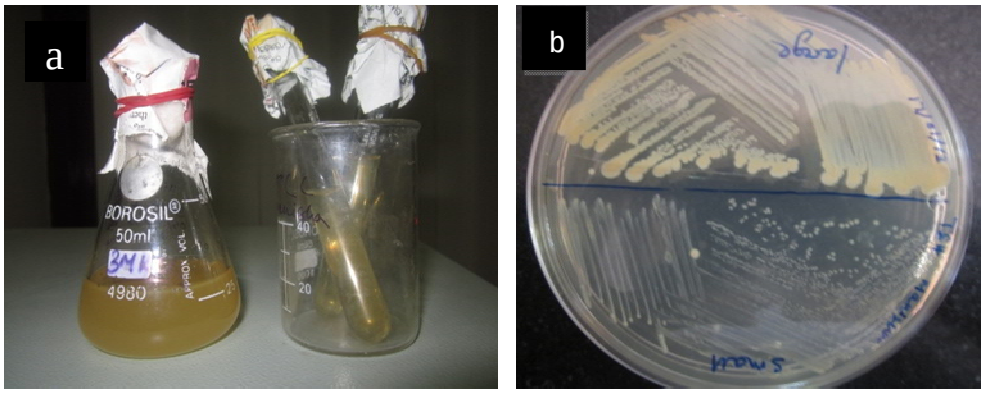

Fig 5a: *Streptococcus pneumoniae MTCC655* in TSB **Fig 5b**: *S. pneumoniae* on TSA plate

5.3 Gram staining

Round oval shaped violet coloured cells in short chains were seen under microscope showing Gram Positive nature of the organism (Fig 6).

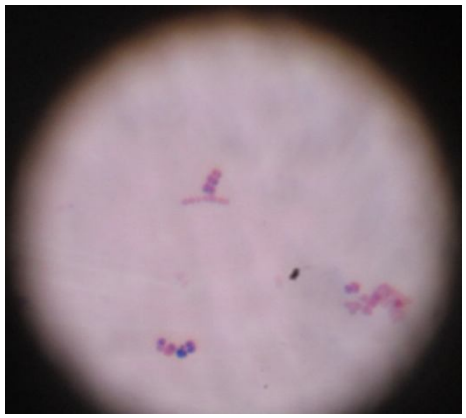

Fig 6: Violet coloured oval cells in chain

5.4 Blood Hemolysis Test

After 12hr of incubation green colour zone was seen around the bacterial colonies on blood agar plate showing alpha hemolysis pattern i.e. incomplete or partial hemolysis (Fig 7).

Fig 7: Alpha hemolysis pattern shown by *Streptococcus pneumoniae* MTCC655

5.5 Bile Solubility Test

After 12hr of incubation a clear solution in the tube containing *Streptococcus pneumoniae MTCC655* was seen indicating lysis of the cells where as the tube containing another bacteria as –ve control had turbid solution showing growth of the organism.

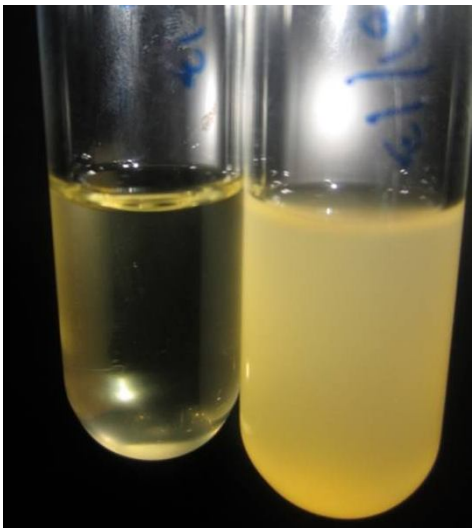

Fig 8: Bile solubility Test

5.6 Biochemical (Sugar Utilization) Test

Among the 12 sugars in the kit only Glycerol showed color change from pink to yellow indicating positive fermentation (Fig 9).

Fig 9: Biochemical (sugar fermentation) Test

5.7 Growth curve of *Streptococcus pneumoniae* MTCC655

The growth curve of *Streptococcus pneumoniae MTCC655* was obtained by plotting the OD against time in excel sheet. The log phase was attained after 12h of inoculation (Fig 10).

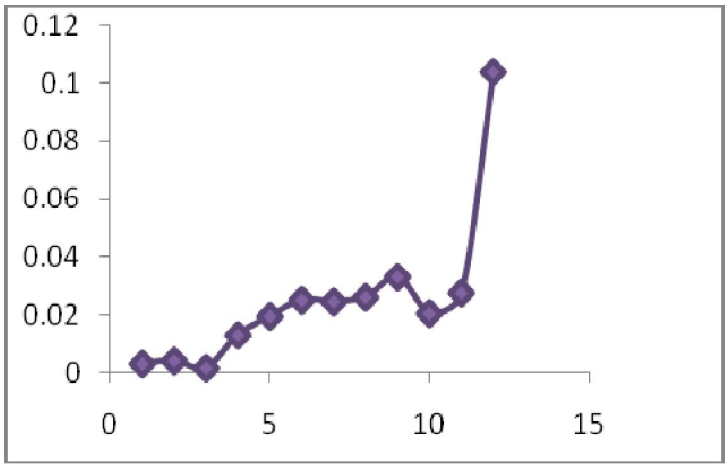

Fig 10: Growth curve of *Streptococcus pneumoniae* MTCC655

5.8 Minimum inhibitory concentration for chloramphenicol

The growth of the organism was observed at different concentrations of chloramphenicol to detect the minimum concentration of antibiotic which can inhibit the growth. The OD of the cultures taken after 12 h incubation was analyzed to detect the growth at different concentrations (Table 2).

Table 2: Growth at different concentrations of chloramphenicol

Concentration of antibiotic	512 µg/ml	256 µg/ml	128 µg/ml	64 µg/ml	32 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml	2 µg/ml	1 µg/ml	MHA + culture	MHA + antibiotic
OD at 595nm 1	0.076	0.077	0.084	0.088	0.110	0.103	0.111	0.116	0.115	0.125	0.117	0.058
OD at 595 nm 2	0.087	0.077	0.096	0.099	0.124	0.136	0.153	0.149	0.171	0.157	0.131	0.056
Mean OD at 595nm	0.082	0.077	0.09	0.094	0.117	0.120	0.132	0.133	0.143	0.141	0.124	0.057

The MIC was found to be 64µg/ml for chloramphenicol**.**

5.9 Antibiotic Susceptibility Test

The zones of inhibitions were seen around the disk (Fig 11). The zone size around the disk was calculated and then that was used for interpretation of the susceptibility (Table 3).

Table 3: Antibiotic Susceptibility (Mean±SD, n=3)

Antibiotic	Mean±SD	Interpretation
Amoxicyllin (AM^{20})	0	AM^{R}
Cefpodoxime (CEP^{10})	17.3±0.1	CEP^{R}
Cephotaxime (CTX^{30})	23±0	CTX^{S}
Chloramphenicol (C^{30})	25±0	C^{S}
Ciprofloxacin (CF^{5})	29±0	CF^{S}
Erythromycin (E^{15})	1.26±0.1	E^{R}
Kanamycin (K^{30})	20.3±0.05	K^{S}
Tetracyclin (T^{30})	20.3±0.05	T^{S}
Vancomycin (V^{30})	13.6±0.05	V^{R}

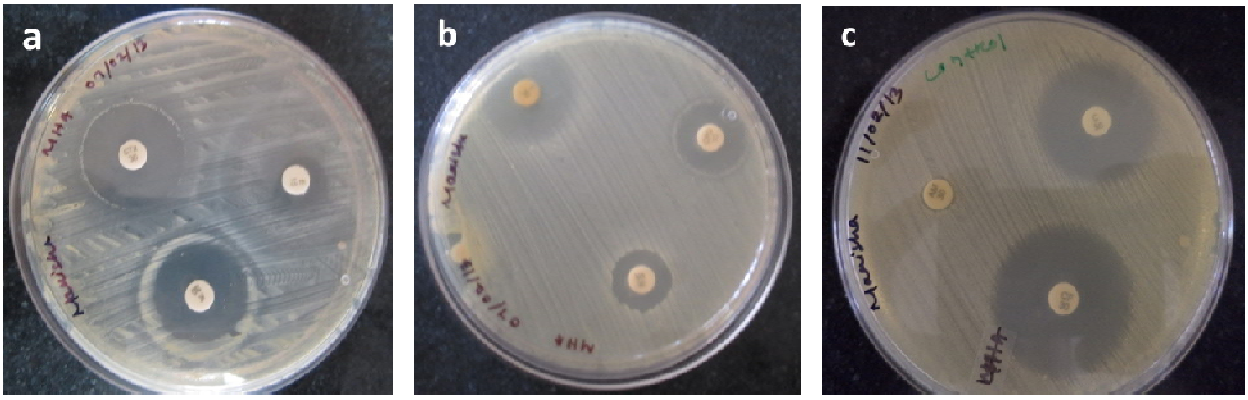

Fig 11 (a, b and c): Antibiotic Sensitivity Test

5.10 In vitro Allolysis Testing

A. By Viable Cell Count

Number of cells present in the culture medium was counted in terms of colony forming units per ml of culture to observe the time at which the cell density reaches 10^7 cells/ml and the possible decrease in number of cells after that point due to allolysis. The CFU/ml was calculated for culture grown in presence and absence of antibiotic at different time intervals (Table 4).

B. Quantitation of Extracellular DNA using Nanodrop

The quantity of extracellular DNA released by the lysis of non-competent cells was estimated using nanodrop so as to observe the in vitro allolysis process both in presence and absence of antibiotic stress. The concentration of extracellular DNA was measured in both the cultures at successive time intervals (Table 5).

Table 4: CFUs on Plates after treatment with antibiotic

Time Interval	With Antibiotic (No of Colonies)	Dilution factor	CFU/ml	without Antibiotic (No of Colonies)	Dilution factor	CFU/ml
0 h	23	10^{-5}	$2.3x10^{7}$	21	10^{5}	$2.1x\ 0^{7}$
2 h	91	10^{-5}	$9.1x10^{7}$	454	10^{5}	$4.54x10^{8}$
4 h	122	10^{-5}	$1.22x10^{8}$	>300	10^{6}	$3.0x10^{9}$
6 h	32	10^{-5}	$3.2x\ 0^{7}$	522	10^{6}	$5.22x10^{9}$
7h	27	10^{-5}	$2.7x10^{7}$	>300	10^{7}	$3.0x10^{10}$
8 h	2	10^{-6}	$2\ x10^{7}$	16	10^{8}	$1.6x10^{10}$
9h	2	10^{-6}	$2\ x10^{7}$	70	10^{9}	$7x10^{11}$
10 h	3	10^{-7}	$3\ x10^{7}$	300	10^{10}	$3x10^{13}$

Table 5: Concentration of extracellular DNA at different time intervals

Time Interval	Conc of DNA ng/µl (withANTIBIOTIC)	Conc of DNA ng/µl (without ANTIBIOTIC)
0 h	236.1	330.1
2 h	194.0	229.6
3h	255.1	227.3
4h	194.2	185.2
5h	140.9	128.9
6h	155.3	121.4
7h	116.7	90.3
8h	167.9	213.3
9h	262.1	200.2

5.11 Amplification of *cibA, cibB, cbpD* and *ply* genes responsible for allolysis at various conditions

The amplification was observed for three genes that play important roles in lysing the non-competent cells and are expressed only in competent cells whereas *ply* is responsible for virulence of the organism. Amplicon bands were seen in lane 4 and 10 (Fig 12).

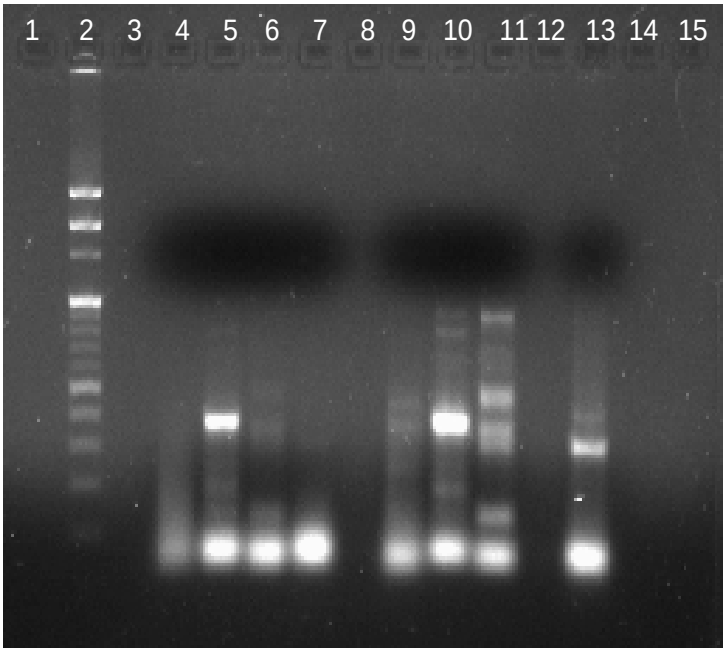

Fig 12: Amplification of *cibA, cibB, cbpD* and *ply:* Lane 2: 100bp ladder, Lane 4 & 9: *cibA*, Lane 5 & 10: *cibB*, Lane 6 & 11: *cbpD*, Lane 7 & 13: *ply*

5.12 Expression level of *cbpD* gene under various conditions by Real Time PCR

The relative expression of the gene *cbpD* which plays a key role in allolysis was observed under stress as well as normal condition to find out the effect of stress on the process. The gene expression was 8 times higher in stress conditions that in normal condition (Fig 13).

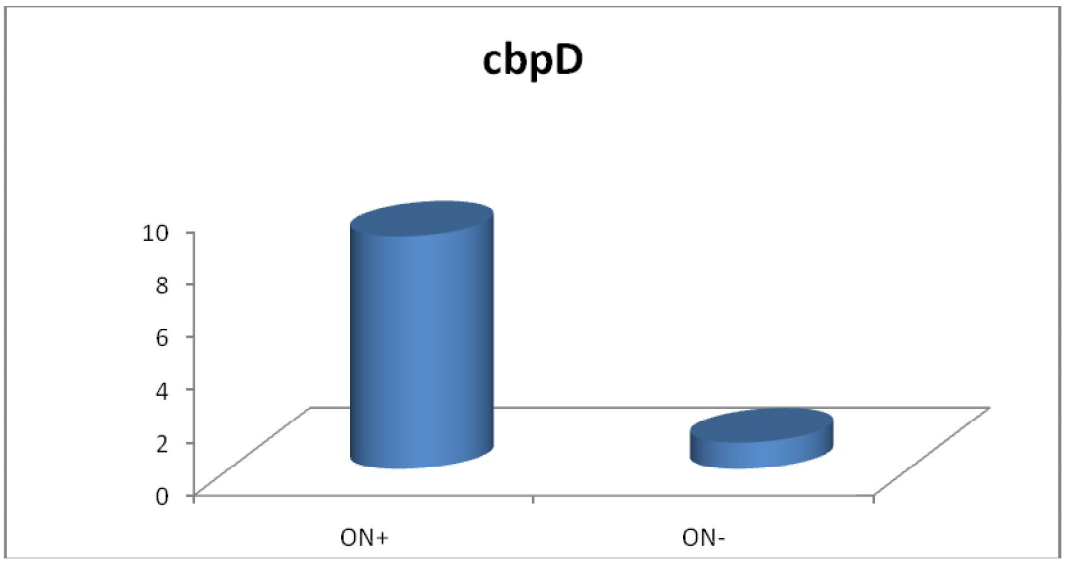

Fig 13: Relative expression of *cbpD* in presence and absence of antibiotic

DISCUSSION

S. pneumoniae has been reported to be responsible for many life threatening diseases like acute sinusitis, otitis media, meningitis, sepsis, endocarditis, brain abscess and many more. Hence, suitable characterization methods should be followed for proper and rapid diagnosis of the causative agent. The phenotypic characterization of the MTCC isolate showed the Gram positive chain of coccus under oil immersion microscope confirming the *Streptococcus* nature of the isolate. The biochemical test results of the isolate showed positive fermentation results for glycerol, glucose, and sucrose which are in accordance to the result obtained by Arbique *et al* (2004). When blood haemolysis test was performed for the study of haemolysis pattern of the isolate, it was found to haemolyse the RBC partially by developing green colorization on the blood agar plates confirming the alpha-haemolysis pattern which is the peculiar characteristics feature of *S. pneumoniae* confirming the nature and identification of the MTCC isolate (Pease et al., 1986). Bile is an inhibiting agent for the growth of *Streptococcus* spp. by degrading the cell wall of Gram positive bacteria. Hence, bile solubility test was performed for proper identification and characterization of the isolate (Merritt and Donaldson,2009). The mechanisms of growth inhibition for Gram positive bacteria by bile salts is well established and was found to inhibit by damaging bacterial cell wall and cell membrane as well as damaging the bacterial DNA. However, the contribution of each process to bacterial death varies across genera and possibly across species. Thus, inhibition of the MTCC isolate by bile salt is well characterized and well established in the current study. The isolate showed perfect growth pattern in nutrient broth medium with somewhat higher lag phase followed by log phase and stationary phase. The present MTCC isolate was found to resist many antibiotic discs including Amoxicyllin (AM^{20}), Cefpodoxime (CEP^{10}), Erythromycin (E^{15}) and Vancomycin (V^{30}). *S. pneumoniae* is well known for its vast range of antibiotic resistance mainly towards the beta lactam group of antibiotics which has been well established in the present study. The antibiotic resistance phenomenon is due to the emergence of resistance genotype by mutation or horizontal gene transfer. However, chloramphenicol was used in this present study as it is considered to be the first line antibiotics effective against both Gram positive and Gram negative pathogens that has been considered to be the protypical broad spectrum antibiotic. When minimum inhibitory concentration of the isolate

was determined following CLSI guidelines by broth micro-dilution technique the isolate was found to tolerate upto 32 µg/mL of the antibiotic tested as the MIC point was observed to be 64 µg/mL. Many reports are there to evidence the higher tolerance limit of *S. pneumoniae* towards chloramphenicol to be > 4 µg/mL and 0.1 to 0.5 µg/mL (Rathod et al., 2012). However, the present isolate could tolerate higher amount of chloramphenicol that any report so far suggesting the more severity for infection and treatment. Though many reports are there for allolysis across the Gram positive and Gram negative isolates, however, the phenomenon was discovered initially in *S. pneumoniae* that has been established in the present study. As allolysis phenomenon leads to the lysis of the cells thus death of the bacteria, viable cell count proves to be suitable screening method for study of allolysis phenomenon. When viable cell count was performed both in presence and absence of antibiotic stress at an interval of 1 hr, in the set of experiment with antibiotic supplementation, after 2 hrs, the number of developed colonies (91 nos.) was found to be less in number in comparison to the negative control (454). Here we get the preliminary idea of the early unset of competence in presence of sub-lethal concentrations of chloramphenicol antibiotic. However, another approach of studying allolysis by quantifying extracellular DNA proves to be quiet useful for this study. When cell lysis occurs, the cellular DNA comes out of the cell to the growth medium, which has been collected and quantified by UV absorption at 260 nm. The study reveals the early unset of allolysis after 3 hr incubation as the antibiotic stress results in higher amount of extracellular DNA (255.1 ng/µL) in comparison to without antibiotic stress (227.3 ng/µl). The genetic analysis of the isolate showed the presence of *cibA*, *cibB*, *cbpD* and *ply* genes in its genomes by giving a clear distinct banding pattern when observed after PCR amplification and agarose gel electrophoresis. As all the above mentioned genes contribute positively for the onset of allolysis in bacteria, the MTCC isolate is proved to be a suitable model for studying allolysis. As autolysins like LytA, LytC and a murine hydrolase choline binding protein D (CbpD) play key roles in hydrolyzing the non-competent cells, analysis of *cbpD* gene expression pattern provided to be useful for a complete understanding of the genetic mechanism and early onset of allolysis under antibiotic stress. The expression pattern shows the up-regulation of *cbpD* gene in presence of antibiotic in comparison to the negative control i.e. without antibiotic stress. Thus, the sub-lethal antibiotic stress plays an important role in inducing allolysis by up regulating the *cbpD* gene expression to a higher level.

CONCLUSION

In 21st century the greatest threat to human beings is the prevalence of multi drug resistant bacteria by nosocomial infections and mere discovery of new antibiotics may not solve the purpose. Hence, suitable alternative medications may be put forward to deal with this noxious problem. In this regard, allolysis phenomenon in many clinical isolate may be given a second thought. As this study clearly demonstrates the early onset of allolysis by *S. pneumoniae* in presence of sub-lethal dose of antibiotic, it may be used for therapeutic purpose. The early onset of allolysis may decrease the number of populations of the antigen, which may be taken care of by the host's immune system. The decrease in time period of allolysis induction from 7-9 hr to 2-3 hr confirmed by viable cell count and extracellular DNA estimation is a significant decrease in time period. Though this study established the phenomenon in case of *S. pneumoniae*, it may be tested against other bacterial pathogens experimentally, suitable clinical trial prior to therapeutic application in large scale. Though the isolate was resistant to a number of antibiotics, it showed allolysis and a decrease in cell population by the sub-lethal dose of antibiotic confirming the superiority of its application for therapeutic use.

FUTURE PROSPECTIVE

- To elucidate the Role of Epigenetics in expression of competent genes
- To investigate the mechanism of immunity to competent cells
- Effect of Allolysis on Host pathogen interaction
- Dvelopment of Drugs targeting Late competent genes
- Discovery of new genes and proteins involved in this process
- Characterization of *comM* and *comW* genes

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